Biochimica et Biophysica Acta 1778 (2008) 1493–1500

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Electrofused giant protoplasts of *Saccharomyces cerevisiae* as a novel system for electrophysiological studies on membrane proteins

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ARTICLE INFO

Article history:
Received 14 January 2008
Received in revised form 15 March 2008
Accepted 20 March 2008
Available online 1 April 2008

Keywords:
Patch clamp
Electrofusion
Yeast
Saccharomyces cerevisiae
Giant protoplast
Channelrhodopsin-2

ABSTRACT

Giant protoplasts of *Saccharomyces cerevisiae* of 10–35 µm in diameter were generated by multi-cell electrofusion. Thereby two different preparation strategies were evaluated with a focus on size distribution and "patchability" of electrofused protoplasts. In general, parental protoplasts were suitable for electrofusion 1–12 h after isolation. The electrophysiological properties of electrofused giant protoplasts could be analyzed by the whole-cell patch clamp technique. The area-specific membrane capacitance (0.66±0.07 µF/cm^2) and conductance (23–44 µS/cm^2) of giant protoplasts were consistent with the corresponding data for parental protoplasts. Measurements with fluorescein-filled patch pipettes allowed to exclude any internal compartmentalisation of giant protoplasts by plasma membranes, since uniform (diffusion-controlled) dye uptake was only observed in the whole-cell configuration, but not in the cell-attached formation. The homogeneous structure of giant protoplasts was further confirmed by the observation that no plasma membrane associated fluorescence was seen in the interior of giant cells after electrofusion of protoplasts expressing the light-activated cation channel Channelrhodopsin-2 (ChR2) linked to yellow fluorescent protein (YFP). Patch clamp analysis of the heterologously expressed ChR2-YFP showed typical blue light dependent, inwardly-directed currents for both electrofused giant and parental protoplasts. Most importantly, neither channel characteristics nor channel expression density was altered by electric field treatment. Summarising, multi-cell electrofusion increases considerably the absolute number of membrane proteins accessible in patch clamp experiments, thus presumably providing a convenient tool for the biophysical investigation of low-signal transporters and channels.

1. Introduction

Saccharomyces cerevisiae is a widely used system for heterologous expression of membrane proteins [1,2]. However, electrophysiological characterisation of transformed yeast cells is practically impossible because of the cell wall. Therefore, application of patch clamp techniques to yeast cells [3] requires first of all the removal of the cell wall by mycolytic enzymes, e.g. zymolyase and glusulase [4]. The small size of the protoplasts (about 4–7 µm in diameter) poses further problems. As pointed out elsewhere [3,5,6], efficient biophysical characterisation of yeast membrane proteins by the patch clamp technique requires the enlargement of yeast protoplasts. Enlarged protoplasts (up to 20 µm in diameter) can be obtained from tetraploid yeast strains and also by subjecting protoplasts to prolonged hypoosmolar stress (<450 mOsm) or supplementing media with 2-deoxy-glucose [5,7]. In an electrophysiological study of a H^+ coupled Cl^- transporter, large multinucleated giant protoplasts were obtained from a *S. cerevisiae* mutant with an interrupted budding cycle [8], with a drawback of a significantly lower protein expression in the mutant strain used.

Multi-cell electrofusion provides a further promising approach for increasing the protoplast size [9,10], without negative effects on the expression level as recently shown for mammalian cells [11]. The electrofusion process includes two essential steps: establishment of an intimate contact between the cells followed by electric field-mediated plasma membrane breakdown in the contact zones of adjacent cells. Non-uniform AC fields are usually used to generate stable cell chains aligned along the field lines resulting in close contacts between cell membranes. Cell alignment results from the translational motion of cells in the non-uniform field, so-called dielectrophoresis [12–15]. The direction of dielectrophoresis is dictated by the complex cell polarizability χ^*, which is defined as $\chi^* = (\varepsilon_c^* - \varepsilon_m^*)/(\varepsilon_c^* + 2\varepsilon_m^*)$, where ε_c^* and ε_m^* are the complex permittivities of cell and medium, respectively. If

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doi:10.1016/j.bbamem.2008.03.015

the real part of χ^* is positive, cells move in the direction of maximum field, usually to the electrodes [16,17]. Membrane breakdown in the contact zone of dielectrophoretically aligned protoplasts and, as a consequence, fusion is triggered by high-intensity DC field pulses of µs-duration. The critical field strength required for breakdown of the yeast protoplast membrane in the contact zone is in the kV/cm range [18,19].

Dense cell suspensions and supracritical DC pulses favour multi-cell electrofusion. At high cell densities, dielectrophoresis leads to the formation of several closely attached chains of protoplasts. Due to the angular dependence of the breakdown voltage, the supracritical DC pulses can induce not only the unidirectional cell fusion within a particular chain but also lateral fusion between adjacent cell chains. The result is the formation of giant multinuclear protoplasts. There are two reports in the literature on the production of giant protoplasts of *S. cerevisiae* by multi-cell electrofusion [10,20], but no electrophysiological evidence was given whether cytoplasmic fusion was completed or membrane fusion was terminated at the level of hemifusion [21]. The latter issue is of key importance because patch clamp studies require complete membrane and cytoplasmic fusion. In this case the strongly enlarged plasma membrane area of giant protoplasts would increase considerably the number of electrically accessible membrane proteins.

In this communication we describe the production of giant protoplasts of *S. cerevisiae* by multi-cell electrofusion, which were suitable for patch clamp studies. Both capacitance measurements and fluorescein diffusion experiments showed that giant protoplasts did not contain plasma membrane-bounded compartments. This finding was also supported by the fluorescence pattern of the yellow fluorescent protein YFP as a part of the fusion protein ChR2-YFP. The potential of giant protoplasts for electrophysiological characterisation of electrogenic membrane proteins expressed in *S. cerevisiae* was demonstrated by the application of the whole-cell patch clamp techniques and the observation of light-induced currents mediated by the cation channel Channelrhodopsin-2, ChR2 [22].

2. Materials and methods

2.1. Molecular biology

The cDNA of ChR2-yellow fluorescent protein was obtained by cutting pcDNA3.1(−)/ChR2-YFP Δbp1098–1300 [22] with BamHI and PmeI. After purification the BamHI–PmeI fragment was subcloned in the pKS1-ST vector (Dualsystems Biotech AG, Zürich, Switzerland) using the cloning site between BamHI and SmaI. Successful construct was confirmed by sequencing. The pKS1-ST vector features a G-418 selection marker (*kan*^{r} gene) and the glucose repressed *ADH2* promoter [23,24]. Once glucose is depleted in the rich medium, the *ADH2* promoter is activated and recombinant protein is expressed. In addition, this expression vector contains an N-terminal Strep-tag followed by an HA epitope-tag, which was used for protein detection by Western blot analysis.

2.2. Cell culture

S. cerevisiae DSY-5 wild type cells (MATa leu2 trp1 ura3-52 his3 pep4 prb1) were grown on YPD medium and chemically transformed as suggested by Dualsystems Biotech AG. Transformants carrying the plasmid pKS1-ST/ChR2-YFP were selected in YPD medium supplemented with 200 µg/ml G-418 (PAA, Pasching, Austria) and 1 µM all trans-retinal (Sigma, Taufkirchen, Germany; liquid cultures only). Wild type and transformed cells were spread from cryo-stocks (dense cell suspension in 25% glycerol in YPD, stored at −80 °C) onto plates (YPD or YPD-G-418, respectively; 2% agar–agar, 30 °C) and stored at 4 °C upon appearance of colonies. These plates were used <3 weeks to inoculate liquid cultures. This was important for good protoplast quality and patch results, as described previously by other authors [3,25,26].

2.3. Western blot/protein expression

2.3.1. Protein-extraction

Cells (1 l culture, shaking 160 rpm, 60 h, 30 °C) were washed and resuspended in breaking buffer [27] (OD_{600nm}=50–55, Nanodrop nd-1000, Wilmington, USA). Acid washed glass beads (0.25–0.5 mm diameter, Roth, Karlsruhe, Germany) were added and cells were decomposed by means of a glass bead beater (Biospec Products, Bartlesville, USA). Cell scrape and beads were pelleted (10,000 g, 10 min, 4 °C) and the supernatant was centrifuged (100,000 g, 1 h, 4 °C). The protein was resuspended (20 mg/ml) in protein buffer [28], homogenised (Dounce-Potter, Potter-Elvehjem, Kontes Glass, Vineland, USA) and stored at −80 °C.

2.3.2. Western blot

Electrophoretic separation of proteins was performed on a 4–12 % bis–tris-gel (NuPAGE^R, Invitrogen, Germany) using a Mini-Cell (MOPS-SDS, 200V; PowerEase 500, Invitrogen, Carlsbad, USA). Proteins were dryly blotted (iBlot, Invitrogen, USA) on a membrane. Either anti-HA antibody (dilution 1:1000; H 6908, Sigma) or anti-GFP antibody (dilution 1:1000; rabbit IgG fraction, Invitrogen, Karlsruhe, Germany) were used for ChR2-YFP-specific immunoreaction. The second immunoreaction (1 h) was performed using an alkaline-phosphatase (AP) conjugated IgG-goat anti-rabbit antibody (dilution 1:3000; Biorad, München, Germany). Blot was incubated in colour developer (Biorad) for <15 min until specific bands became visible. Scanned images were subjected to graphical processing (CorelPaint/Draw 11, Corel, Ottawa, Canada).

2.4. Protoplast preparation

S. cerevisiae DSY-5 wild type cells were grown (25 ml, shaking 220 rpm, 30 °C) for 24 h, whereas transformed cells were harvested in the stationary phase 48–60 h after inoculation (OD_{600nm}=1.0–1.2). Trans-retinal was added 15 h before harvesting.

2.4.1. Protocol 1

Cells were washed with H_2O, resuspended in preincubation buffer (50 mM EDTA and 35 mM β-mercaptoethanol, adjusted to pH 9.0) and shaked for 15 min (30 °C, 140 rpm). After two washing steps in washing solution I (1.2 M sorbitol, 50 mM EDTA, pH 7.5) and washing solution II (1.2 M sorbitol, 50 mM Tris, pH 7.5) cells were resuspended in 5 ml enzyme solution consisting of 1.2 M sorbitol, 50 mM Tris, pH 7.5, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 10 mg/ml albumin fraction V, 5 U/ml zymolyase 100 T (MP Biomedicals, Aurora, USA), 9.9 U/ml glucuronidase plus 1.8 U/ml sulfatase (Glusulase, PerkinElmer, Boston, USA). Cells were incubated in a 25 cm^2 cell culture flask (140 rpm, 30 °C) for 3 h or 15 h over night. In the latter case, digestion mix was complemented by 10% YPD-Medium, 200 U/ml penicillin plus 20 µg/ml streptomycin (100× penicillin/streptomycin, Invitrogen, Germany), 1 µg/ml ergosterol [29] and 2 µg/ml aculeacin (Sigma). Ergosterol was added as a stabilizer of fungal membrane [30,31] and aculeacin was used to avoid regeneration of the cell wall [32].

2.4.2. Protocol 2

This protocol was performed according to Bertl et al. [3] with some modifications. Protoplasting buffer contained 0.125 U zymolyase 100 T and 25 mg/ml albumin fraction V. After 45 min digestion (140 rpm, 30 °C) cells were resuspended in protoplast buffer (containing additional 0.6 % glucose). Separation of protoplasts from remaining cell wall material and undigested yeast cells was achieved by sagging for >1 h at 4 °C.

In both protocols, successful digestion of cell walls was proved microscopically by spherical shape [33] of isolated protoplasts and also by analyzing their swelling behaviour and bursting after decreasing the osmolarity to less than ~370 mOsm [34]. Prior to electrofusion, protoplasts were centrifuged (500 g, 2 min, 4 °C), washed two times and resuspended in the fusion medium (0.1 mM calcium acetate, 0.5 mM magnesium acetate and 800 mM sorbitol). Protoplast suspensions (OD_{600nm}=0.40–0.45) were stored at −1 °C (cooling device Ch-100, Lab4You, Berlin) and used within 6 h.

2.5. Electrofusion

Protoplast suspension (2–3 µl) was electrofused in a fusion chamber (two electrode wires glued in parallel on a microslide 100 µm apart) using a modified Multiporator (Eppendorf, Hamburg, Germany). Cell alignment was achieved by application of an alternating field of 2 MHz frequency and 30 s duration. AC field strength was set to 450 V/cm (Protocol 1) or 550 V/cm (Protocol 2). The aligned cells were subjected to 4 consecutive rectangular field pulses of 10 kV/cm strength and 20 µs duration (Protocol 1) or 12 kV/cm strength and 25 µs duration (Protocol 2). Upon the fourth pulse the AC field was applied again for 20 s with field strength reduced to 300 V/cm. Afterwards 5 µl fusion medium was added and protoplasts were allowed to relax at room temperature for 5 min before the sample was transferred to the perfusion chamber.

2.6. Patch clamp

Patch clamp experiments were performed as described previously [11] with some modifications. Whole-cell currents were recorded with an Axopatch 200B amplifier coupled to a DigiData 1440 interface (Molecular Devices Corporation, Union City, USA), low-pass filtered at 2 kHz and digitized at a sampling rate of 10 to 100 kHz. Data recordings were controlled by the Software Clampex 10.0 (Molecular Devices), data analysis was performed using Clampfit 10.0 (Molecular Devices) and Origin 7.5 (OriginLab Corporation, Northampton, USA). A special perfusion chamber was developed to separate cell debris from (electrofused) protoplasts. A broad gentle stream of buffer perfused through the chamber removing small particles whereas patchable protoplasts remained. Solution-exchange was completed within 2 min. Protoplasts prepared by Protocol 1 (see section 2.4.1.) were cooled in a bath solution to 14–17 °C in order to facilitate giga-seal establishment. A diode pumped solid-state laser (λ=473 nm, Pusch OptoTech, Baden-Baden, Germany) allowed blue-light illumination, which was controlled by a uniblitz shutter (VCM-D1 controler and VS25 series, Vincent associates, Rochester, USA) connected to the DigiData 1440 interface. Pipettes (1 µm tip, 5–8 MΩ, GB150F-8P glass; Scienceproducts GmbH, Hofheim, Germany) were pulled on a three-stage horizontal puller (DMZ Universal Puller, Zeitzinstrumente, Martinsried, Germany). The stray capacitance (capacitance of pipette, pipette-holder and headstage of the amplifier) was

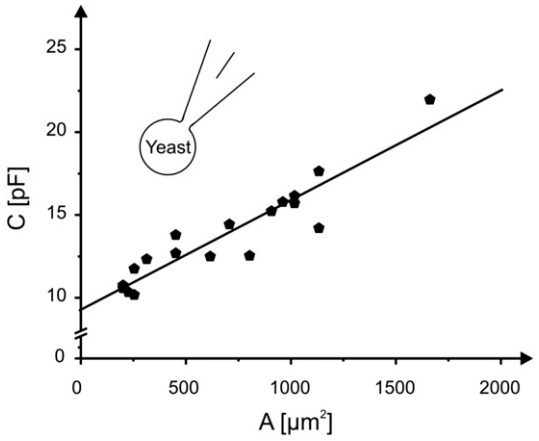

Fig. 1. Plot of the cell capacitance (including stray capacitance) *versus* the membrane area of electrofused giant protoplasts of *S. cerevisiae*. The linear regression to the data gave a slope of 0.66 ± 0.07 µF/cm^2 and a *Y*-intercept of 9.3 ± 0.5 pF. Note that the values for membrane area were calculated from the microscopically determined cell diameters, by assuming spherical geometry. Membrane areas of 150 µm^2 and 1500 µm^2 correspond roughly to cell diameters of 7 and 22 µm, respectively.

determined to be 9.0 ± 0.2 pF (n = 4). Pipette solution contained 10 mM EGTA, 110 mM KCl, and 5 mM $MgCl_2$. The sealing buffer and bath solution contained 140 mM NaCl, 5 mM $MgCl_2$ and 10 mM $CaCl_2$. To generate higher signal amplitudes a guanidine$^+$-buffer was used, containing 320 mM CH_5N_3Cl, 5 mM $MgCl_2$ and 10 mM $CaCl_2$. All buffers were complemented by 10 mM HEPES and pH was adjusted to 7.4 (pipette and sealing buffer), 6.0 (guanidine$^+$-buffer) and 5.0 (bath solution) by appropriate addition of NaOH or HCl. The osmolality was adjusted with sorbitol to 700 mOsm/kg.

Overpressure of 15 mbar was applied to the patch pipette to avoid occlusion of the pipette tip with suspended particles. Before attaching the pipette tip to unfused protoplasts (following Protocol 2), a sudden underpressure (−15 mbar) was applied and, subsequently, the protoplast was sucked to the pipette tip. To achieve a giga-seal resistance underpressure was applied between −15 and −40 mbar, in some cases up to −120 mbar. In case of giant protoplasts (prepared by Protocol 1) the pipette tip was approximated to the membrane until a slight deformation of the cytoplasma membrane was observed. Subsequently, underpressure of −5 mbar was applied. During this procedure the use of poly-D-lysine coated coverslips was helpful to immobilise giant protoplasts on the surface. Formation of a giga-seal resistance was achieved within 5–15 min by gentle increase of underpressure. In contrast to the patch clamp approaches on unfused protoplasts, disruption of giant protoplasts occurred by applying underpressure lower than −40 mbar. Whole-cell configuration was achieved by an electrical pulse under a slight underpressure (−15 mbar) or by applying a transient change in underpressure to −100 mbar within 1 s.

2.7. Fluorescence microscopy

Successful cell wall digestion and regeneration of the cell wall by protoplasts were investigated using fluorescent staining on *S. cerevisiae* DSY-5 wild type cells with FITC-labeled ConA (Sigma) [35] and calcofluor white (fluorescent brightener 28, Sigma) [36,37]. Fluorescence excitation was induced by using a 50 W HBO installed in an axioskop (Zeiss, Jena, Germany) with a 60-fold LD objective and fluorescein specific filterset AF105-2 and the calcofluor specific filter set XF06 (Omega optical, Brattleboro, USA). An eventual internal separation of multiple fusion entities into membrane-bound compartments was investigated by adding sodium fluorescein (Sigma) to the patch-pipette solution [11]. Differences in signal intensity of fluorescein were presented by false colours. Confocal laser images were obtained with a Leica TCS-SP5 microscope (63.0 × 1.40 Oil objective) using a 514 nm Argon laser excitation and 525–600 nm detection. Images were processed by Imaris 5.72 (Bitplane, Zürich, Switzerland).

3. Results

3.1. Production and characteristic features of giant protoplasts

Spherical protoplasts prepared by both Protocols 1 and 2 could be exposed for 5–6 h to fusion medium (800 mOsm sorbitol solution) without any adverse side effects under ice-cooling conditions. However, exposure of protoplasts prepared by Protocol 1 to hypotonic 400 mOsm sorbitol solution resulted in cell bursting, whereas protoplasts prepared by Protocol 2 were stable for several hours in this medium. Exposure to a 100 mOsm sorbitol solution resulted in bursting of both types of protoplasts within 10 min.

Application of four short DC pulses to dielectrophoretically aligned protoplasts frequently resulted in multiple fusion events. For both types of protoplasts, electrofusion conditions were optimized independently as described in section 2.5. After electric field treatment according to Protocols 1 and 2, 10–60% and 3–10% of electrofused protoplasts, respectively, exhibited a diameter ranging between 15 and 25 µm. In general, Protocol 2 yielded only rarely giant protoplasts larger than 25 µm, whereas in the case of Protocol 1 electrofused protoplasts were often even larger than 30 µm. Occasionally, electrofused protoplasts with a diameter of up to 50 µm were observed. However, it was unclear, whether the electrofusion process in the latter case resulted in homogeneously fused giant entities or was terminated at the level of hemifusion due to insufficient giga-seal conditions in the patch clamp experiments on such electrofused protoplasts.

3.2. Resistance and membrane characteristics of parental and giant protoplasts

In the case of giant protoplasts prepared by Protocol 2, cell-attached configuration was achieved in about 50% of trials within 5 min. However, transfer to the whole-cell configuration was successful only in less then 5% of protoplasts. In contrast, only 20% of the trials with the giant protoplasts produced by Protocol 1 yielded cell-attached configuration within 10 min, but subsequently >75% were effectively transferred to the whole-cell configuration.

In view of these results, only giant protoplasts prepared by Protocol 1 were used for the following patch clamp experiments. Seal-resistances of 2–20 GΩ in the cell-attached configuration were routinely obtained 1–4 h after electrofusion if protoplasts were used within 12 h after cell wall digestion. For protoplasts with a diameter <25 µm, resistances of 1–10 GΩ were recorded in the whole-cell configuration, whereas resistance values below 1 GΩ were exclusively obtained from protoplasts of 30–50 µm in diameter. The membrane area of protoplasts was calculated from their diameters, by assuming spherical geometry. The specific conductivity of the membrane of giant protoplasts of up to 25 µm in diameter was in the range of 23–44 µS/cm^2, which

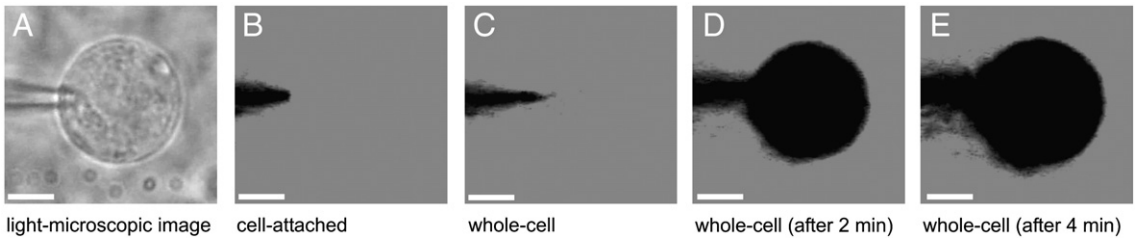

Fig. 2. Light (A) and fluorescence microphotographs (B–E) of a patched giant protoplast (*S. cerevisiae* DSY5-wild type, diameter about 22 µm). The patch pipette was filled with a solution containing fluorescein. Black colour in images B–E corresponds to the maximum fluorescence intensity. No pipette-to-cell directed diffusion of fluorescein took place in the cell-attached configuration (B). Fluorescence images C, D and E were taken immediately, 2 and 4 min after establishment of the whole-cell configuration, respectively (for further details, see text). White bars correspond to 10 µm.

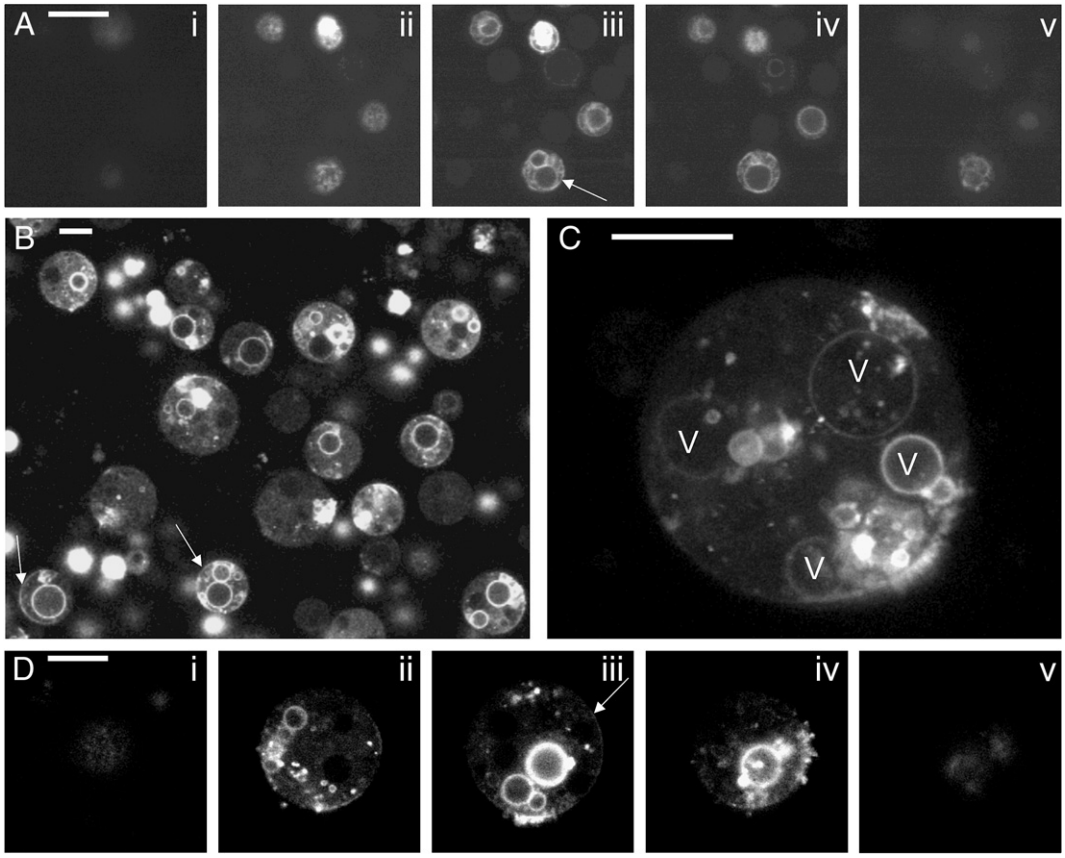

Fig. 3. Typical confocal fluorescence images of various samples of yeast protoplasts expressing ChR2-YFP fusion protein. Images in (A, i–v) show five serial confocal cross-sections through a sample of none-fused protoplasts. Arrows (A-iii, B, D-iii) indicate YFP fluorescence in the plasma membrane. Image in (B) shows a typical suspension of electrofused protoplasts. A giant protoplast with a diameter of ~20 µm containing several vacuoles (V) is shown in (C). Confocal cross-sections (D, i–v) through a giant protoplast (20 µm diameter) reveals that in vacuolar membranes YFP was expressed at much higher levels than in the plasma membranes (arrow in D-iii). Images (B–D) were taken 10 min after electrofusion. White bars correspond to 10 µm.

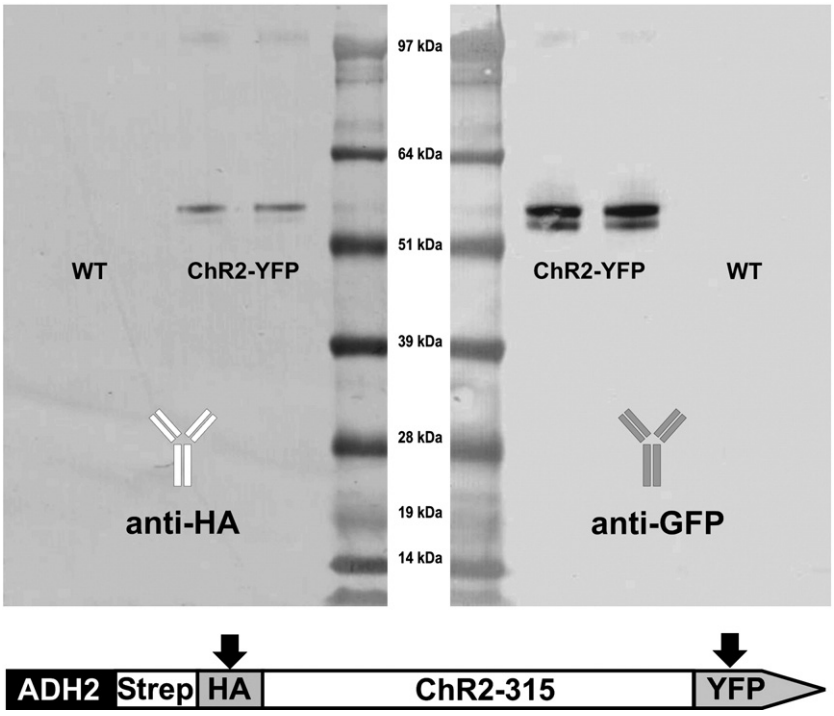

Fig. 4. Western blot of membrane proteins from *S. cerevisiae* DSY-5 wild type (WT) cells and ones transformed with vector pKS1-ST/ChR2-YFP. The fusion protein provides two epitopes (black arrows): the HA-tag and the YFP protein. Using both anti-HA and anti-GFP antibodies a clear band (58–60 kDa) and a weak band (55 kDa) were detected in membrane proteins from transformed cells but not from wild type cells.

corresponds to the specific conductivity of ~13 μS/cm^2 obtained at electrofused HEK293 cells [11]. Fig. 1 shows the membrane capacitance data for the electrofused *S. cerevisiae* protoplasts (measured in the whole-cell configuration) plotted against the membrane area (for cell diameters ranging between 8 and 25 μm). The data points could be approximated quite well by a straight line, whose slope yielded a value for the area-specific membrane capacitance of 0.66 ±0.07 μF/cm^2. The *Y*-intercept of the regression line represents the stray capacitance of 9.3±0.5 pF, which is in good agreement with that measured from lipid-clogged patch pipettes in control experiments (see section 2.6).

3.3. Fluorescein-mediated fluorescence uptake in giant protoplasts

The absence of plasma membrane-bounded compartments in the giant protoplasts was confirmed in patch clamp experiments using pipette solution containing fluorescein. As clearly seen in Fig. 2B, fluorescein did not enter the giant protoplast (22 μm diameter) in the cell-attached configuration. Significant dye uptake occurred only after membrane breakthrough and establishment of the whole-cell configuration (Fig. 2C and D). Four minutes later (Fig. 2E), the fluorescent dye was distributed uniformly within the giant protoplast.

3.4. ChR2 expression in yeast protoplasts

The light-activated cation channel ChR2 was expressed as fusion protein with YFP linked to its C-terminal end. Together with unfused yeast protoplasts (Fig. 3A) typical confocal fluorescence images of fusion products are given in Fig. 3B–D. The plasma membrane boundaries of the individual protoplasts seem to have totally disappeared upon formation of the giant protoplasts. In general, no clusters of unfused protoplasts were observed (Fig. 3B). As clearly indicated by differences in the fluorescence intensity, the expression level of the fusion protein ChR2-YFP differed in both single and giant protoplasts. However, the fusion product in Fig. 3D-iii shows a homogeneous expression of ChR2-YFP in its plasma membrane (white arrow). In general, the highest expression density was localised not in the plasma membrane but obviously in the vacuolar membranes (V).

Heterologous expression of the fusion protein ChR2-YFP was also proved by Western blot analysis (see Fig. 4). Two different antibodies (anti-HA and anti-GFP) were used for ChR2-YFP-specific immunoreaction. Analysis of membrane proteins extracted from yeast cells transformed with construct pKS1-ST/ChR2-YFP showed a broad band in the range of 58–60 kDa. A narrow band in the range of about 55 kDa was also detected with both antibodies, which is most probably due to incomplete glucosylation of the protein as revealed by analysis of tunicamycin-treated cultures (data not shown). In contrast no GFP- or HA-specific band was detectable in membrane proteins derived from DSY5 wild type cells.

3.5. Whole-cell recordings of ChR2 on parental and electrofused giant protoplasts

Functional activity of ChR2 was analyzed by patch clamp techniques in both parental and electrofused giant protoplasts. Fig. 5A illustrates a typical whole-cell recording on a parental protoplast subjected to inward-directed strong guadinine$^+$ as well as H$^+$-gradients. Blue-light illumination of the cell evoked inwardly-rectifying photodependent currents under these conditions. The kinetics of the photodependent currents measured in *S. cerevisiae* protoplasts, were almost identical to those measured in other expression systems: A simple three-state-model [22] can be used to describe the photocycle of ChR2. The time constant τ_1 reflecting the opening of the channel was determined to be <0.5 ms. It should be emphazised that the time resolution was limited by shutter opening velocity and by using a 2 kHz low-pass filter. Time constants τ_2 and τ_{off} were 18±1 ms and 7±1 ms,

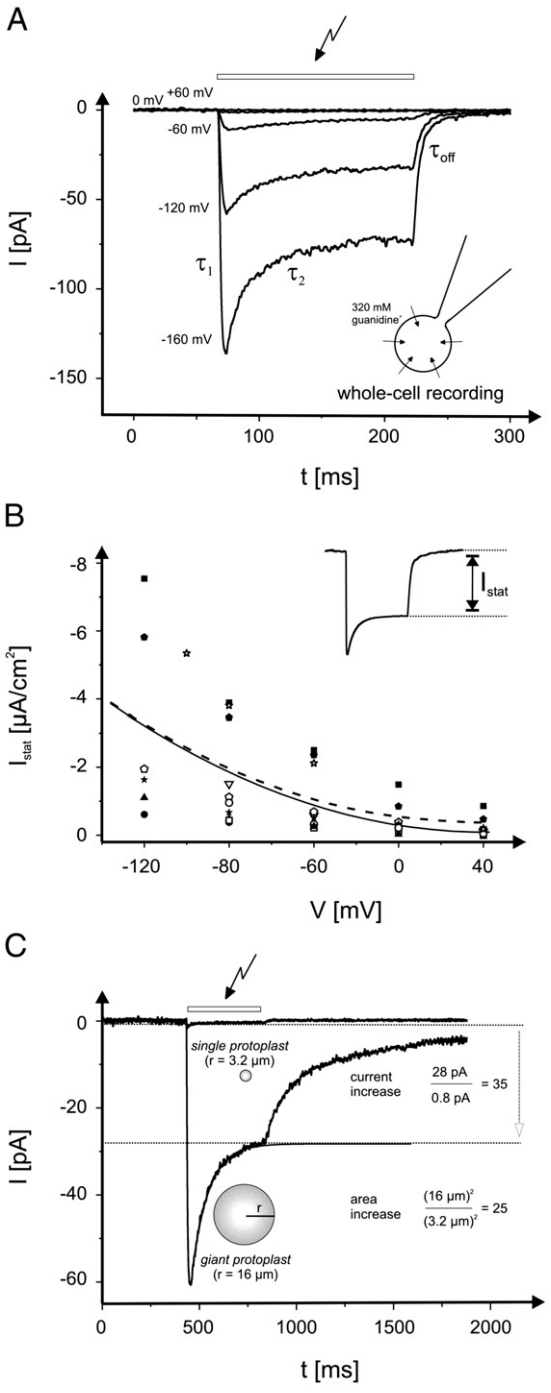

Fig. 5. Whole-cell patch clamp recordings on parental and giant protoplasts of *S. cerevisiae* expressing ChR2-YFP. (A) Typical whole-cell recording from a parental protoplast (7 μm diameter) subjected to strong inward-directed guanidine$^+$- and H$^+$-gradients. The applied voltage protocol ranged from +60 mV to −160 mV as indicated by inscription (holding potential was 0 mV). Downwardly directed arrow signifies illumination by blue light. τ_1, τ_2 and τ_{off} represent the time constants, which are characteristic for ChR2 (see text). (B) Current-voltage relationship of the ChR2-mediated area-specific stationary current obtained at single protoplasts (filled symbols; diameter 6.6±1.1 μm; n=5) and electrofused giant protoplasts (open symbols; diameter 21.8±6.2 μm, n=5). Mean values are presented by dotted and continuous curves, respectively. (C) Typical whole-cell trace from a single protoplast (diameter 6.4 μm) in relation to one of an electrofused protoplast (diameter 32 μm) under similar experimental conditions (holding potential of 0 mV, inward-directed H$^+$ and Na$^+$-gradients). The inscriptions denote the relationship between the surface area and current increases (see text).

respectively (at −160 mV). Using the simplified state-model of ChR2 introduced by Nagel et al. [22] the ratio of molecules in the open state can roughly be determined to be around 50% (under these conditions). This is in good agreement with the corresponding data for HEK293 cells expressing ChR2 under similar experimental conditions (data not shown). At −160 mV the area-specific current density varied between 6.7 and 40.9 μA/cm^2 (protoplasts, n=4), suggesting a relatively low expression level of ChR2 in the plasma membrane as well as its strong variation among the individual giant protoplasts (For comparison, area-specific current densities in HEK293 cells were larger by at least one order of magnitude [11]).

The fluorescence microphotographs in Fig. 3A and B reveal large variations with respect to the ChR2-YFP fluorescence related to the plasma membrane. In agreement with these microscopic observations, the light-activated current densities also proved to be subject to great variations (Fig. 5B) in both parental and electrofused protoplasts expressing ChR2. It should be noted, however, that the mean area-specific current densities measured in electrofused protoplasts (Fig. 5B, continuous curve) over a range of holding potentials upon stationary illumination were similar to the corresponding data of parental ChR2-expressing cells (dotted curve).

Compared to parental protoplasts, the ChR2 mediated current amplitudes in giant protoplasts were substantially increased, by a factor roughly matching the surface area enlargement. This issue is illustrated in Fig. 5C by two typical voltage-clamp traces obtained from a non-fused protoplast (diameter 6.4 μm) and a giant electrofused protoplast (32 μm). By assuming spherical geometry, the surface area ratio of the two protoplasts was ~25, which was somewhat lower than the corresponding ChR2-mediated current ratio of ~35. The observed discrepancy can be explained by the large variations in the ChR2 expression density mentioned above (see Figs. 3 and 5B). It has also to be noted that measurements presented in Fig. 5C were conducted under more physiological conditions (ion permeability: $H^+ >> Gua^+ >> Na^+$ [22]), as compared to the steep guanidine$^+$-gradient used in the experiments shown in Fig. 5A. For that reason, the current amplitude in Fig. 5C is lower than in Fig. 5A.

4. Discussion

The results presented here clearly show that multi-cell electrofusion provides an effective tool for large-scale production of giant yeast protoplasts suitable for electrophysiological studies. Electrofusion requires close membrane contact between dielectrophoretically aligned protoplasts, which can be significantly improved under hypotonic conditions. Compared to isotonic media, hypotonic solutions yield generally higher numbers of viable fusion products in a variety of cell systems, including mammalian, plant and yeast cells [20,38,39]. As discussed elsewhere [10,12,13], hypotonic cell swelling improves membrane contacts due to smoothing of the cell surface, and also facilitates cell fusion due the increased mobility of membrane components and temporary dissolution of the cytoskeleton. Hypotonic swelling also enhances both cell alignment and membrane breakdown simply because the dielectrophoretic force and induced membrane voltage scale linearly with the cell volume and radius, respectively. For mammalian cells, which are commonly capable of withstanding extreme hypotonic swelling, osmolalities 3–4 times lower than the physiological one are optimal for electrofusion [11,14,16,38].

Isolated protoplasts of *S. cerevisiae*, are apparently less tolerant to hypotonic stress than mammalian cells. Upon acute hypotonic exposure, the water influx (e.g. via aquaporins [40,41]) leads to rapid swelling of *S. cerevisiae* protoplasts in accordance to a near-ideal osmometer [34]. Unfortunately, protoplasts frequently burst upon excessive swelling in strongly hypotonic media. Based on the results of an earlier study [20] and our preliminary data, the osmolality of 800 mOsm was found to be optimum with respect to both osmotic stability of protoplasts and electrofusion yields. Both fusion protocols evaluated here were based on 800 mOsm medium. It is important to stress that reproducible results were only achieved if protocols were accurately followed. In both protocols three-dimensional fusion between protoplasts, which had been dielectrophoretically aligned in chains by a MHz-field, was induced by a train of four DC field pulses. The optimum intensity of the DC pulses depended on the method of protoplast preparation.

In general, Protocol 2 is based on previous methods introduced by Bertl et al. [3]. Prominent feature of this protocol is a strongly lowered enzyme concentration for the cell wall digestion in contrast to other protocols [20,42–44]. According to Bertl et al. [3] this mild enzyme treatment does not dissolve cell walls, but cracks them and releases protoplasts with surfaces of sufficient cleanness for successful application of the patch clamp technique [45]. However, the "patchability" of electrofused protoplasts produced by the Protocol 2 was poor, as compared to the giant protoplasts generated by high-enzyme Protocol 1. It can be speculated that the observed characteristics of protoplasts prepared by Protocol 2 were due to the remaining cell wall structures and further ramifications of the plasma membrane surface [46–50], which obviously did not prevent the sealing process of attached micropipettes, but may have hindered the electrofusion process of the membranes [44]. The above considerations suggest that the protoplast preparation by the "high-enzyme" protocol facilitates both electrical and pressure-induced breakthrough of the membranes, thus improving the establishment of the whole-cell configuration.

Protoplast enlargement is beneficial if the whole plasma membrane of the giant protoplast is accessible to electrophysiological investigations. The formation of giant protoplasts without plasma membrane-bounded compartments was apparently successfully performed in the present study. This conclusion is supported by the following data. First, the area-specific capacitance value of 0.66 μF/cm^2 (Fig. 1) is similar to that measured in yeast cells using an electrorotation approach [51]. Second, confocal fluorescence microscopy images of giant protoplasts expressing ChR2-YFP clearly demonstrate the complete fusion of plasma membranes of individual protoplasts upon electrofusion (Fig. 3). Third, the membrane-impermeable dye fluorescein [52] diffused uniformly from the patch pipette into the cell and distributed evenly in the cytosol of giant protoplasts (Fig. 2). This is only possible if there are no boundaries of original plasma membranes of the individual cells in the interior of the giant cells. The fact that the plasmalemma of giant protoplasts is impermeable to fluorescein was also demonstrated as no fluorescein diffusion occurred in the cell-attached patch clamp mode (Fig. 2B).

Electric fields used to manipulate cells are known to induce changes in the bilayer assembly and to upregulate genes. [53,54]. Nevertheless field treatment and the following cascade of fusion events apparently did not change the essential physiological functions of the homogeneously fused giant protoplasts. Among others, field-treated protoplasts showed regeneration of cell walls. This was evidenced by a positive ConA-FITC staining of field-treated protoplasts, which were incubated at 30 °C in YPD medium for more than 24 h (data not shown). Accordingly, Weber and Berg [44] describe that the regeneration of protoplasts is unchanged after electrical field treatment. Furthermore, as suggested by the non-altered electrophysiological properties of ChR2-YFP in the giant protoplasts (see Fig. 5), the electric field treatment exerted no adverse effects on the membrane integrity and protein function. According to Nagel et al. [22] the typical inwardly-rectifying current-voltage characteristics of ChR2 were observed in the case of both parental and giant protoplasts (Fig. 5B). Furthermore, also the kinetics of the ChR2-mediated currents was unchanged compared to that reported for *Xenopus laevis* oocytes and parental HEK293 cells [22]. Blue-light illumination of protoplasts evoked a quick rise (τ_1) of the current subsequently decaying within milliseconds (τ_2) to a steady-state current (I_{stat}), which disappeared (τ_{off}) when light was switched off (see the current traces recorded at hyperpolarising voltages; Fig. 5A).

Most importantly, the protein density of ChR2-YFP in the plasma membrane was not affected by electrofusion, as confirmed by confocal

fluorescence microscopy (Fig. 3) and independently by comparative electrophysiological measurements of light-activated currents on parental and giant protoplasts (Fig. 5B and C). It is noteworthy that the expression of ChR2 was only sufficient for patch clamp experiments, if cells were taken from the late stationary phase. In general, the expression of ChR2-YFP was low in the plasma membrane of the yeast protoplasts, as compared to the expression of ChR2 in HEK293 cells (assuming a similar single channel conductance). Huge photo-dependent currents (Fig. 5A) from protoplasts were only observed in the presence of strong inwardly-directed gradients of both guanidine$^+$- and H^+-ions, but not under more physiological conditions (Fig. 5B and C). A sufficient signal-to-noise ratio under latter conditions was only yielded by using the electrofused protoplasts.

Summarizing, the results presented here for yeast protoplasts are in line with those recently reported for electrofused mammalian cells [11]. The substantial increase in the number of functional membrane molecules provided by the large plasma membrane area of electrofused giant yeast protoplasts allows patch clamp studies of membrane proteins even under low-signal conditions. Thus, electrofused giant protoplasts are expected to offer a useful experimental system for the electrophysiological characterisation of ion translocating systems if either expression level or ion turn-over rate of the molecule is low.

Acknowledgements

We thank A. Pustlauck and M. Vöth for their excellent technical support (MPI of Biophysics and the University of Frankfurt am Main, respectively). We also thank J. Reichert and A. Gessner (MPI of Biophysics and the University of Würzburg, respectively) for their excellent fine engineering support. We further thank Dr. R. Reuss (University of Würzburg) for the critical reading and correction of the manuscript. This work was supported by a grant from the BMBF to EB (0313369B).

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